INTRODUCTION

Variability is a prerequisite for selection programme, it is necessary to detect and document the amount of variation existing within and between populations. DNA marker based fingerprinting can distinguish species rapidly using small amounts of DNA and therefore can assist to deduce reliable information on their phylogenetic relationships. DNA markers are not typically influenced by environmental conditions and therefore can be used to describe patterns of genetic variation among plant populations and to identify duplicated accessions within germplasm collections (Jubera et al., 2009).

Advances in molecular biology techniques have provided the basis for uncovering virtually unlimited number of DNA markers. The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism (Bardakci, 2001). Various approaches are available for DNA fingerprinting such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), simple sequence repeats (SSRs) and randomly amplified polymorphic DNA (RAPD).

Currently, the restriction fragment length polymorphism (RFLP) assay has been the choice for many species to measure genetic diversity and construct a genetic linkage map (Botstein et al., 1980). However, an RFLP assay which detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridisation, is in general time consuming and laborious (Bardakci, 2001).

Among these, RAPD is an inexpensive and rapid method not requiring any information regarding the genome of the plant, and has been widely used to ascertain the genetic diversity in several plants. RAPD requires only small amount of genomic DNA and can produce high levels of polymorphism and may facilitate more effective
diversity analysis in plants and it provides information that can help to define the distinctiveness of species and phylogenetic relationships at molecular level (Ganesh et al., 2007). The simplicity and applicability of the RAPD technique have captivated many scientists interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement of cloning, sequencing or any other form of molecular characterisation of the genome of the species in question (Bardakci, 2001).

The standard RAPD technology utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR (William et al., 1990).

Isolating high quality DNA is essential for molecular research. Polysaccharide contamination is a common problem in higher plant DNA extraction. DNA samples are often contaminated with melicera colloidal hyalosome, which is almost insoluble in water or TE buffer. This can affect manipulation, inhibit enzyme reactions (Fang et al., 1992; Porebski et al., 1997; Schlink and Reski, 2002), and hinder the downstream work in molecular biology research. DNA samples are not stable for long time storage (Lodhi et al., 1994; Sharma et al., 2002).

Over the last decade, polymerase chain reaction (PCR) technology has become a widespread research technique and has led to the development of several novel genetic assays based on selective amplification of DNA (Erlich, 1989). This popularity of PCR is primarily due to its apparent simplicity and high probability of success. Unfortunately, because of the need for DNA sequence information, PCR assays are limited in their application. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (Williams et al., 1990; Welsh and McClelland, 1990).

At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the amplification complementary sequences in the template genomic DNA and produce
discrete DNA products if these priming sites are with an amplifiable distance of each
other. The profile of amplified DNA primarily depends on nucleotide sequence
homology between the template DNA and oligonucleotide primer at the end of each
amplified product.

Nucleotide variation between different sets of template DNAs will result in the
presence or absence of bands because of changes in the priming sites. Recently,
sequence characterised amplified regions (SCARs) analysis of RAPD polymorphisms
(Paran and Michelmore, 1993; Bardakci and Skibinski, 1999) showed that one cause of
RAPD polymorphisms is chromosomal rearrangements such as insertions/deletions.
Therefore, amplification products from the same alleles in a heterozygote differ in
length and will be detected as presence and absence of bands in the RAPD profile. The
profile of RAPD bands is similar to that of low stringency minisatellite DNA
fingerprinting patterns and is therefore also termed RAPD fingerprinting. On average,
each primer directs amplification of several discrete loci in the genome so that alleles
is not distinguishable in RAPD patterns. In other words, it is not possible to distinguish
whether a DNA segment is amplified from a locus that is heterozygous or homozygous
RAPD markers are therefore dominant.

The RAPD technique has received a great deal of attention from population
geneticists (Hedrick, 1992) because of its simplicity and rapidity in revealing DNA
level genetic variation and therefore has been praised as the DNA equivalent of
allozyme electrophoresis (Skibinski, 1994).

The antioxidant activity in each district sample was also carried out to check
whether the variations are found using DPPH assay and super oxide radical scavenging
assay.

**MATERIALS AND METHOD**

**Fruits**

*M. calabura* fruits were collected from 10 districts of Tamilnadu namely
Namakkal, Salem, Trichy, Tirupur, Erode, Karur, Kanchipuram, Madurai, Dharmapuri,
and Nilgris for the analysis of comparison of antioxidant activity among the individuals and their genetic diversity.

**Extraction for antioxidant activity**

Firstly, the fruits (100 g) were blended and extracted with methanol for 2 hours at room temperature in an orbital shaker; then the extract was centrifuged and the supernatant was collected and dried using a rotary evaporator at 40°C. The dried methanol extract was obtained, and various concentrations of the extracts were prepared from the resultant extract to determine the *in vitro* antioxidant activity.

**Evaluation of antioxidant activity of *M. calabura* fruit extract**

1. **DPPH radical scavenging assay**

   The effect of extracts on DPPH radical was determined using the method of Szabo *et al.* (2007). Different concentrations of the extracts (500, 400, 300, 200, 100 µg/ml) were prepared and subjected to antioxidant tests. To 1 ml of each of the extracts, 5 ml of 0.1mM methanol solution of DPPH was added, vortexed, followed by incubation at 27ºC for 20 min. The control was prepared without any extract and absorbance of the sample was measured at 517 nm using UV/VIS Spectrophotometer (ELICO) using methanol to set 0. The ability to scavenge DPPH radical was calculated by the following equation:

   $$\text{DPPH radical scavenging activity (%) = \frac{[\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}]}{\text{Abs}_{\text{control}}} \times 100}$$

2. **Super oxide anion scavenging activity**

   Measurement of super oxide anion scavenging activity of *M. calabura* extracts was based on the method described by Liu *et al.* (1997). Super oxide radicals were generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). 3 ml of sample solutions at different concentrations were mixed with 1 ml of NBT (156µM) and 1 ml of NADH (468 µM). The reaction started by adding 0.1ml of phenazine metho sulphate (PMS) solution (60µM) to the mixture. The reaction mixture was incubated at 25ºC for 5 min, and the absorbance at 560 nm was measured against blank. Decreased absorbance of the reaction mixture
indicates increased super oxide anion scavenging activity. The percentage inhibition of super oxide anion generation was calculated using the following formula:

\[
\text{Inhibition of super oxide generation (\%) } = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100.
\]

**Genetic diversity analysis:**

**Plant material:** Plant tissue (fruit) was sampled from 10 *M. calabura* individuals representing 10 districts of Tamilnadu, India. Namely Namakkal, Salem, Trichy, Tirupur, Erode, Karur, Kanchipuram, Madurai, Dharmapuri, Nilgris for genetic diversity analysis.

**DNA Extraction for genetic diversity analysis:** Plant tissue (fruit) was sampled from 10 *M. calabura* individuals representing 10 districts of Tamilnadu, India. Total DNA was extracted according to the procedure of Dellaporta *et al.* (1983). The quality and quantity of DNA were checked by analytic electrophoresis in a 1% agarose gel containing ethidium bromide (0.5 mg ml⁻¹) in 1×TBE as described by Sambrook *et al.* (1989).

**Primers and PCR Assays:** Totally 10 primers were used for our current studies. The primer was obtained from Sigma, India and used in the PCR. Primer sequences used in the study was given in Table 1 with their sequences.

Isolated DNA samples were amplified by PCR conditions described by Jones *et al.* (2002) with minor modifications. After first step of primer screening, amplifications were performed with little modifications to the procedure of Williams *et al.* (1990). Each amplification was carried out with (20 µl) contained 2 µl of template DNA (Genomic DNA), 2 µl of 10 X PCR buffer, 1.5 µl of 25 pmol of each of the primers, 1 µl of 25 mM of each deoxynucleotide triphosphate and 1 µl of Taq DNA polymerase (Conc. 3U/µl) and 12.5 µl of molecular grade water. A brief spin was given to settle down the materials then tubes were kept in thermocycler (Genei). After initial denaturation at 94°C for 6 min, the samples were subjected to 40 cycles of denaturation at 94°C for 2 min, annealing at 44°C for 1 min and extension at 72°C for
1 min. A final extension was performed at 72°C for 10 min. Following PCR, the reaction mixtures were analyzed by electrophoresis on a 1% Agarose gel, containing ethidium bromide (0.2 mg/ml), in the presence of an appropriate DNA molecular weight marker. The amplification bands were observed and under UV Transilluminater and photographed. Molecular weights of amplified products were estimated using 1 Kb Ladder (GIBCO-BRL, France). PCR-RAPD assays were performed in duplicate.

Table 1. The primers used for the genetic diversity analysis

<table>
<thead>
<tr>
<th>S.No</th>
<th>Name of Primer</th>
<th>Primer sequence (3’ to 5’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPAG-07</td>
<td>CACAGACCTGT</td>
</tr>
<tr>
<td>2</td>
<td>OPAG-O8</td>
<td>AAGAGCCCTC</td>
</tr>
<tr>
<td>3</td>
<td>OPAG-O9</td>
<td>CCGAGGGGTT</td>
</tr>
<tr>
<td>4</td>
<td>OPV-O6</td>
<td>ACCGCCAGGT</td>
</tr>
<tr>
<td>5</td>
<td>OPV-O9</td>
<td>TGATCCCGTC</td>
</tr>
<tr>
<td>6</td>
<td>OPV-2O</td>
<td>CAGCATGGTC</td>
</tr>
<tr>
<td>7</td>
<td>OPX-O1</td>
<td>CTGGGACACGA</td>
</tr>
<tr>
<td>8</td>
<td>OPX-17</td>
<td>GACACGGACC</td>
</tr>
<tr>
<td>9</td>
<td>OPP-O1</td>
<td>GTAGCACTCC</td>
</tr>
<tr>
<td>10</td>
<td>OPP-O4</td>
<td>GTGTCTCAGG</td>
</tr>
</tbody>
</table>

**Agarose Gel Electrophoresis**: Agarose gel electrophoresis was carried out in a horizontal submarine electrophoresis unit. Thirty ml of 1 % Agarose gel was prepared with 1X TBE buffer (do not mix) and heated the content to get up to clear solution for casting Agarose gel. After cooling the solution, 7 µl of staining dye solution was added into the casting system. The gel was allowed to solidify, and then carefully disassembled from the casting system without disturbing the wells and placed in 1X TBE buffer filled electrophoresis tank (the buffer level should be above gel). 5 µl of genomic sample DNA mixed with 2 µl of gel loading dye and then loaded to gel and simultaneously 3 µl of DNA marker was loaded in the nearby well. The power card terminals were connected at respective positions, the gel was run at 50 V, till the gel loading dye migrate more than half the length of gel. Then the unit was switched off and was visualized under UV Transilluminator for the isolated DNA.
### Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>50mM Tris</td>
<td>0.605 gm</td>
</tr>
<tr>
<td>10 mM EDTA</td>
<td>0.372 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>PH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

### Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 N NaOH</td>
<td>0.8 gm</td>
</tr>
<tr>
<td>SDS</td>
<td>1 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
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</tbody>
</table>

### Solution C

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>3.2M Potassium Acetate</td>
<td>31.4 gm</td>
</tr>
</tbody>
</table>

### Solution D

100 %Isopropyl Alcohol

### 1X TBE Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>10.8 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>5.5 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.92 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>PH</td>
<td>8.3</td>
</tr>
</tbody>
</table>

### 1X TE Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>10.8 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.92 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>PH</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**Sample loading dye:** Sucrose 40% and bromophenol blue (0.25%) were mixed with sterile double distilled water.

**Ethidium bromide:** Ethidium bromide (10mg) was mixed with 10 ml of sterile distilled water. (It is carcinogenic and should be treated or handled accordingly).
Data Analysis

The DNA profiles were manually scored directly from gel photographs and only repetitive bands, i.e., those occurring in the two duplicates, were considered. The fragments produced by each primer were treated as characters and numbered sequentially. Genotypes were scored for the presence (1) or absence (0) of all polymorphic bands.

Besides, the RAPD banding patterns transformed into a binary matrix. The genetic distance matrix was estimated using the Genedist (version 3.572c) program based on the formula developed by Nei and Li (1979). Cluster analysis was made using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) by PHYLIP software (Phylogeny Inference Package, version 3.5c) [Phylip (1995) and TreeView (Win32, version1.5.2) (Page, 1996)].

RESULT

In our study, the fruits of M. calabura collected from various districts of Tamilnadu were evaluated for its variation in their antioxidant activity. In order to determine the antioxidant activity, two well established in vitro protocols were applied. The IC_{50} values were obtained for the tested assays and given in Table 2.

Table 2. Showing the IC_{50} Value of DPPH assay and SOR scavenging assays

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of district</th>
<th>IC_{50} VALUE µG/ML</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DPPH</td>
</tr>
<tr>
<td>1</td>
<td>Namakkal</td>
<td>78</td>
</tr>
<tr>
<td>2</td>
<td>Salem</td>
<td>91</td>
</tr>
<tr>
<td>3</td>
<td>Trichy</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>Tirupur</td>
<td>88</td>
</tr>
<tr>
<td>5</td>
<td>Erode</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>Karur</td>
<td>85</td>
</tr>
<tr>
<td>7</td>
<td>Kanchipuram</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>Madurai</td>
<td>76</td>
</tr>
<tr>
<td>9</td>
<td>Dharmapuri</td>
<td>72</td>
</tr>
<tr>
<td>10</td>
<td>Nilgiris</td>
<td>95</td>
</tr>
</tbody>
</table>
From the results obtained it was clearly notified that there was a moderate variation in the antioxidant activity of the fruits collected from different places. This variation in the antioxidant activity may be due to environmental factors also. The environmental factors include climate, moisture content, temperature, water availability, pH, stress and other parameters also. Thus it was clearly understood that the abiotic factors also play a major role in the activity of the plant.

It is well known that the composition of secondary plant metabolites depends on numerous factors, particularly plant chemotype, cultivation conditions, and harvesting time. Remarkable variations in chemical composition can be observed in the plants grown in wild and uncontrolled environment. (Williams et al., 1990)

All the ten individual samples *M. calabura* were examined for RAPD genetic markers with ten decamer primers. Molecular marker data in conjunction to morphological data could be highly useful in precise differentiation and relatedness among the cultivars. Each RAPD product was assumed to represent a single locus and datas were scored as (1) for presence and (0) for its absence. In this study random primers were used to determine the genetic diversity by PCR amplification. The primers were subjected to optimized conditions for PCR, were applied to all sample DNA. The amplified fragments ranging from 100 bp to above 1000bp. These primers exhibited discriminatory band patterns among the plant. The bands produced were shown in Plate 1. This data was utilised for further computations.

DNA fingerprint was developed for all the samples. Among the 10 primers tested, only five generated unambiguously reproducible bands, others did not produced distinctive bands. Primer 1, 3, 4 and 5 produce number of bands in all samples. The pictures of RAPD PCR gel amplified by primers were shown in Plate 1a – 1j. The number of markers dedected by each primer depends on primer sequence and the extent of variation is genotype. In our current studies most of the primers did not produced bands in 9th and 10th samples of the plant. In our RAPD analysis various types of band patterns were obtained. Some common bands were observed to be present in large number of the isolates.
Plate 1

RAPD analysis of *M. calabura* using different primers

![Image of RAPD analysis results]

a. Primer 1 (OPAG – 07)

Lane 1 – Namakkal  
Lane 4 – Tirupur  
Lane 7 – Kanchipuram  
Lane 10 – Nilgiris

Lane 2 – Salem  
Lane 5 – Erode  
Lane 8 – Madurai  
M - Marker

Lane 3 – Trichy  
Lane 6 – Karur  
Lane 9 – Dharmapuri
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c. Primer 3 (OPAG – 09)

d. Primer 4 (OPV - 06)

Lane 1 – Namakkal       Lane 2 – Salem       Lane 3 – Trichy
Lane 4 – Tirupur        Lane 5 – Erode       Lane 6 – Karur
Lane 7 – Kanchipuram    Lane 8 – Madurai     Lane 9 – Dharmapuri
Lane 10 – Nilgiris      M - Marker

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Lane 1 – Namakkal       Lane 2 – Salem       Lane 3 – Trichy
Lane 4 – Tirupur        Lane 5 – Erode       Lane 6 – Karur
Lane 7 – Kanchipuram    Lane 8 – Madurai     Lane 9 – Dharmapuri
Lane 10 – Nilgiris      M - Marker

e. Primer 5 (OPV – 09)

f. Primer 6 (OPV – 20)
Lane 1 – Namakkal       Lane 2 – Salem       Lane 3 – Trichy
Lane 4 – Tirupur        Lane 5 – Erode        Lane 6 – Karur
Lane 7 – Kanchipuram   Lane 8 – Madurai       Lane 9 – Dharmapuri
Lane 10 – Nilgiris      M - Marker
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i. Primer 9 (OPP – 01)

j. Primer 10 (OPP – 04)

Lane 1 – Namakkal  Lane 2 – Salem  Lane 3 – Trichy
Lane 4 – Tirupur  Lane 5 – Erode  Lane 6 – Karur
Lane 7 – Kanchipuram  Lane 8 – Madurai  Lane 9 – Dharmapuri
Lane 10 – Nilgiris  M - Marker
The lowest molecular weight bands observed in most of the samples (150 bp) and above 1000bp molecular weight bands were observed from number of samples. In this RAPD analysis the highest molecular weight bands were observed in first primer’s pattern. In our investigation number of polymorphic bands was observed.

The genetic distance was computed and dendrogram was constructed considering all the individuals which produced different band patterns, (Figure 1 - 5). The genetic distance was calculated and used for cluster analysis and dendrogram construction. The dendrogram for the pooled data separated genotypes into two major clusters. The genotypes which were found in one cluster were further divided into sub clusters. The data showed no specific grouping of the genotype based on the diversity analysis.

Figure 1. Dendrogram of primer 2 along with genetic distance
Figure 2. Dendogram of primer 3 along with genetic distance

Figure 3. Dendogram of primer 4 along with genetic distance
Figure 4. Dendogram of primer 5 along with genetic distance

Figure 5. Dendogram of primer 6 along with genetic distance
Considering the dendrogram constructed from the pooled data, it is evident that some set of primers chosen for the study were not able to group the genotypes into phenotypically intended category such as morpho-physiological characters. Such a moderate variation in the species like *M. calabura* could be due to the few introductions that have spread across the country primarily.

**CONCLUSION**

Monitoring the antioxidant properties and the genetic diversity of *M. calabura* fruits collected from various districts of Tamilnadu was the main task of this chapter. It was found that all the methanol extract of *M. calabura* fruits collected from various districts were antioxidatively active and showed variations in the activity measured by DPPH assay and super oxide radical scavenging assays. The present study constitutes a prerequisite for the development of a molecular method suitable in the genetic polymorphisms. RAPD markers have found a wide range of applications in gene mapping, population genetics, molecular evolutionary genetics and plant and animal breeding. This is mainly due to the speed, cost and efficiency of the RAPD technique to generate large numbers of markers in a short period compared with other methods. Therefore, RAPD technique can be performed in a moderate laboratory for most of its applications (Bardakci, 2001).

**REFERENCES**


Studies on Antioxidant and Pharmacological activities of Montina calabura Linn. (Elaeocarpaceae) fruits


